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Genomic Characterization of Human Astrovirus Type 6 Katano Virus and the Establishment of a Rapid and Effective Reverse Transcription–Polymerase Chain Reaction to Detect All Serotypes of Human Astrovirus

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We previously reported that human astrovirus type 6 (HAstV T6) was the etiologic agent of a large-scale outbreak of acute gastroenteritis that occurred in 1991 in Katano City, Osaka, Japan [Oishi et al., 1994]. The two representative strains, Katano virus K23 and K24, have been analyzed by sequencing the open reading frame 2 (ORF2) region after amplification by reverse transcription–polymerase chain reaction (RT-PCR). The ORF2 region of HAstV T6 strains, including K23, was found to be about 20 bp smaller than those of other types. There was 94% nucleotide sequence identity and 95% amino acid sequence identity between K23 and K24, with the Oxford strains belonging to HAstV T6. The high homology of the ORF2 region between the Katano and Oxford strains shows intratype genomic stability, irrespective of time and place of virus isolation. Comparing sequences of ORF2 of different HAstV serotypes, we established a rapid and highly sensitive detection system for HAstV types using RT-PCR with the AC230/AC1′ primer set designed from the 5′-terminal end region of ORF2. This RT-PCR system seems very useful in detecting at least two different viruses in a single PCR test tube using AC230/AC1′ in addition to the NV81/82, SM82 primer sets. Thus, our rapid and effective detection system may contribute to the epidemiologic characterization of astrovirus infections as well as Norwalk-like viruses. ***J. Med. Virol. 61:125–131, 2000.***

KEY WORDS: human astrovirus type 6; capsid coding region; open reading frame 2; sequence; reverse transcription–polymerase chain reaction; Norwalk-like virus

INTRODUCTION

Human astroviruses (HAstVs) are 28-nm viruses possessing single-stranded RNA of positive polarity as their genome; they were first detected in stool specimens obtained from infants and children with diarrhea by Appleton and Higgins [1975] using electron microscopy (EM). HAstVs were given their name by Madeley and Cosgrove [1975] owing to the star-shaped appearance of some of the particles. Initially discovered astroviruses were classified as Caliciviridae but now are classified as Astroviridae because of their different genomic conformation and characterization [Monroe et al., 1993]. Monroe et al. [1993] and Carter and Willcocks [1996] reported that the astrovirus RNA genome is about 6.8 kb and contains a poly-A tract at the 3′-end consisting of three open reading frames (ORFs), of which ORF2 encodes a capsid structural protein.

Using new and more sensitive methods, such as reverse transcription–polymerase chain reaction (RT-PCR) [Noel et al., 1995; Mustafa et al., 1998; Traore et al., 1998], hybridization assays, enzyme-linked immunosorbent assays (ELISAs) [Noel et al., 1995; Kriston et al., 1996], viral isolation techniques [Lee and Kurtz, 1981], and EM [Monroe et al., 1991], studies have indicated that human astroviruses are a common and important causative agent of acute gastroenteritis. In addition, these methods have contributed to the clarification of the infection routes of human astroviruses

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[Myint et al., 1994; Traore et al., 1998] and have changed their epidemiologic profile [Glass et al., 1996; Maldonado et al., 1998; Shastri et al., 1998].

Human astroviruses are classified into eight serotypes based on results of immunofluorescence assays (IFAs) using rabbit antisera [Kurtz and Lee, 1984]. Epidemiologic studies have shown that serotype 1 (T1) is the major causative agent of gastroenteritis [Konno et al., 1982; Koopmans et al., 1998; Lee and Kurtz, 1994; Mitchell et al., 1999], though there also have been large outbreaks of acute gastroenteritis associated with HAstV T6 [Oishi et al., 1994] and T3 [Belliot et al., 1997].

In this preliminary study we attempted the development of a highly sensitive PCR system to detect HAstV in stool specimens using newly designed primer pairs derived from the 3′ end of the ORF2 genome region. Using our new primer set (AC4/AC6), a unique, clear band on the electrophoresis gel could be ascribed as a product of T6, since the ORF2 region is about 20 bp smaller than the ORF2 regions from other serotypes. These results prompted us to characterize genetically T6 strains, especially the Katano virus, which has been detected in a large-scale gastroenteritis outbreak that might be associated with school lunches contaminated with this virus [Oishi et al., 1994].

Since 1997, the Department of Food Sanitary Science of the Ministry of Health and Welfare of Japan has been notified of diagnosed cases of viral, food-borne gastroenteritis. It has recommended the development of diagnostic systems for the detection of astroviruses as well as Norwalk-like viruses (NLVs), since both are important causal agents of acute gastroenteritis. In this report, we discuss the genomic characterization of the HAstV T6 strains newly isolated in Japan and the development of an effective method to detect all serotypes of HAstV using a new primer set, AC230/AC1′, designed from the conservative N-terminal region of HAstV ORF2. In addition, we report that multiplex RT-PCR with the AC230/AC1′ primer set and the other primers for detection of NLVs was established and that this system was useful for rapid laboratory diagnosis of viral gastroenteritis.

MATERIALS AND METHODS

Viruses

Reference strains of HAstV T1 through 7 were kindly provided by Dr. Ando of the Centers for Disease Control. The viruses were passaged in LLC-MK2 cells or CaCo-2 cells, and the Oxford type 6 strain (OxAs T6) was used as the control virus. Fecal specimens K23 and K24, obtained from patients with gastroenteritis during the Katano outbreak in 1991, tested positive for astrovirus particles [Oishi et al., 1994]. The K23 and K24 strains, a sixth passage in CaCo-2 cells (K23-6), and another newly isolated strain collected from sporadic cases of diarrhea in Japanese children (3–7 years old) were tested. These isolated strains from sporadic cases were detected by EM from 1988 to 1995. These strains were than passaged four or seven times in CaCo-2 cells, and the serotype was determined by ELISA with anti-HAstV rabbit serum. Other RNA viruses, including rotavirus (SA11), NLV (KY89/89/J), and hepatitis A virus (KRM238), were used for testing the specificity of the PCR assay.

Extraction of Viral RNAs

Ten percent (w/v) stool suspensions in phosphate-buffered saline were centrifuged at 15,000 rpm for 10 min at 4°C, and the supernatant was used for viral RNA extraction. Viral RNAs were extracted using ISOGEN-LS (Nippon Gene, Japan) from 250 μL of the supernatant or virus-cultured fluid. The procedures for extraction were performed according to the manufacturer's protocol. The RNA pellet was resuspended in 30 μL of diethyl pyrocarbonate–treated water.

Reverse Transcription–Polymerase Chain Reaction Amplification of Katano Strain ORF2

An amplification contig map of cDNA of the ORF2 gene and the 3′ end of the ORF1b gene from OxAs T6 was made. The full-length ORF2 region of the Katano strains was amplified in fragments using RT-PCR, with nine primer sets designed to overlap each side of the sequence of OxAs T6 by about 50 bp.

The final concentrations of the RT master mix were as follows: 1× Pyrobest buffer containing 1 mmol/L Mg^{2+} at pH 8.3 (Takara Shuzo, Japan), 1 mmol/L of each dNTP, 2.5 μmol/L antisense primer, 1 U/μL RNase inhibitor, and 2.5 U/μL reverse transcriptase (MuLV; Perkin-Elmer Applied Biosystems, Oak Brook, IL). Viral RNA was incubated at 55°C for 5 min and quenched on ice; then 0.5 μL viral RNA was added to the master mix. Viral RNA was reverse-transcribed at 42°C for 30 min, incubated at 99°C for 5 min to destroy reverse transcriptase activity, and cooled at 5°C for 5 min. Before PCR, cDNA was preheated at 94°C for 1 min and amplified using 35 cycles of denaturation at 94°C for 10 sec and annealing at 50°C for 30 sec, with an extension at 72°C for 3 min using the DNA Thermal Cycler (Perkin-Elmer). The final concentrations for PCR were as follows: 1× Pyrobest buffer containing 1 mmol/L Mg^{2+} , at pH 8.3 (Takara Shuzo), 0.2 mmol/L of each dNTP, 0.5 μmol/L of each primer, and 2.5 U/100 μL Pyrobest DNA polymerase (Takara Shuzo). The PCR product was subjected to 2% agarose gel electrophoresis and observed under ultraviolet illumination after staining with ethidium bromide. The molecular marker used was 100-bp DNA Ladder (BioLabs, U.K.) or pGEM DNA Markers (Promega, Madison, WI).

Sequence Analysis of Nucleotide and Deduced Amino Acid of ORF2

The nine PCR products were purified with a PCR purification kit (QIAGEN, Germany) and sequenced directly from both the 5′- and 3′-terminal ends of ORF2 using the PRISMTM Dye-Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer). From the sequences of the nine products, a single-stranded cDNA sequence was deduced. To investigate genetic homology, the full-length sequences of nucleotide and predicted amino

TABLE I. Sequences of Primers Used for Reverse Transcription–Polymerase Chain Reaction and Probe

Primer probe	Position of 5′ nucleotide	Polarity	Primer sequence, 5′ to 3′
AC4	2065	+	GACGAAGCGGACAGGTTTGA
AC6	2444	–	GCTTCTGATTAAATCAATTTTAAA
AC230	230	–	GGTTTTGGTCCTGTGACACC
AC1′	1	+	ATGGCTAGCAAGTCTGACAAG
ACom-probe	174		TCAACGTGTCCGTAACATTATCAATAA
NV81	4875	–	ACAATCTCATCATCACCATA
NV82	4545	+	TCACTATGATGCAGATTA
SM82	4543	+	CCACTATGATGCAGATTA

The positions of AC primers and ACom-probe were from the 5′ end of HAstV ORF2.

acid of the K23 ORF2 were compared with the data in the EMBL/GenBank of HAstVs using DNASIS software.

Reverse Transcription–Polymerase Chain Reaction to Detect for All Serotypes of HAstV

Primer pairs were prepared using the 5′ end and 3′ end of the ORF2 region (sequences shown in Table I). The concentrations in the master mix were as follows: 10 mmol/L Tris-HCl at pH 8.3, 2.5 mmol/L $MgCl_2$, 1 mmol/L of each dNTP, 2.5 μmol/L of antisense primer, 1 U/μL RNase inhibitor, and 2.5 U/μL reverse transcriptase (MuLV). RT reactions were the same as those of the ORF2. The final concentrations in the PCR premix were as follows: 10 mmol/L Tris-HCl at pH 8.3, 1 mmol/L $MgCl_2$, 0.8 mmol/L of each dNTP, 0.5 μmol/L of sense primer, and 2 U/100 μL of Ampli*Taq* DNA Polymerase (Perkins-Elmer). cDNA was amplified using 30 cycles of denaturation at 94°C for 30 sec, followed by annealing at 55°C for 30 sec with an extension at 72°C for 1 min. The concentration of $MgCl_2$ was examined from 0.5 mmol/L to 3 mmol/L. Other RNA viruses were tested for confirmation of specificity.

Multiplex Reverse Transcription–Polymerase Chain Reaction of Human Astroviruses and Norwalk-like Virus

The K23 strain of HAstV and the KY89/89/J strain of NLV were also tested to determine if single-tube detection by RT-PCR using a mixture of the AC230/AC1′ primer set with the NV81/82, SM82 primer set was feasible (Table I) [Wang et al., 1994; Sasaki et al., 1996]. This primer set, designed by Sasaki et al. [1996] and modified from the primers reported by Wang et al. [1994], is common for detection of NLV in Japan. As cited for RT-PCR to detect all HAstV, 1 μmol/L of AC230 primer and 2.5 μmol/L of NV81 primer were added to the RT master mix. After RT and preheating, PCR was carried out for 40 cycles of denaturation at 94°C for 30 sec, followed by annealing at 45°C for 30 sec and extension at 72°C for 1 min. These reactions were modified from the procedures reported by Jiang et al. [1992]. The final concentrations of premix for PCR were the same as for the procedure mentioned above, with the addition of 0.5 μmol/L of NV82 and SM82 primers. The concentrations of the AC230/AC1′ primer set were changed 0.5 μmol/L to 0.2 μmol/L.

Southern Hybridization

PCR products were identified by Southern hybridization using 5′ end labeling oligonucleotides with a digoxigenin-3-o-methylcarbonyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester probe (Table I).

Nucleotide and Amino Acid Sequence Accession Number

Sequence data from this study have been deposited with the EMBL/GenBank data libraries. The accession numbers for the viruses are L23518 (OxAs T1), L13745 (OxAs T2), AF117209 (T3), 33883 (OxAs T4), U15136 (T5), Z46658 (OxAs T6), Z66541 (T8), ABO13618 (K23), ABO31031 (K23-6), and ABO31030 (K24).

RESULTS

ORF2 Nucleotide Sequence Analysis of Katano Viruses

The Katano strains K23 (2,548 bp), K23-6 (2,418 bp), and K24 (2,548 bp), including the ORF1b region, have been sequenced. All ORF2 regions of these strains contain 2,337 nucleotides, predicting 778 amino acids. No differences were found in the molecular size of K23, K23-6, K24, and the OxAs T6 reference strain. The ORF2 regions of the Katano strains showed 94% nucleotide identity and 95% amino acid identity with OxAs T6. The identity between K23 and K24 was 99.8% in 2,548 bp. The sequence of K23-6 showed a few changes in three nucleotides and two amino acids without sequence deletion. These changes were found at 2250 (G to C), 2270 (C to T), and 2308 (T to C) of the nucleotide sequence.

Comparison of the nucleotide sequence of K23, K23-6, and K24 with those of reference strains of T1 to T8 (except T7) indicated that the nucleotide identity was 62% to 74%. Sequences at the 5′ end region were well conserved, whereas those near the 3′ end were significantly more variable. Predicted amino acid sequences from ORF2 of the Oxford reference T1, T6, and K23 indicated that the genomes of T6 and T1 were significantly similar between amino acid residues 1 and 416, even though the T6 strain had a 24 bp (eight amino

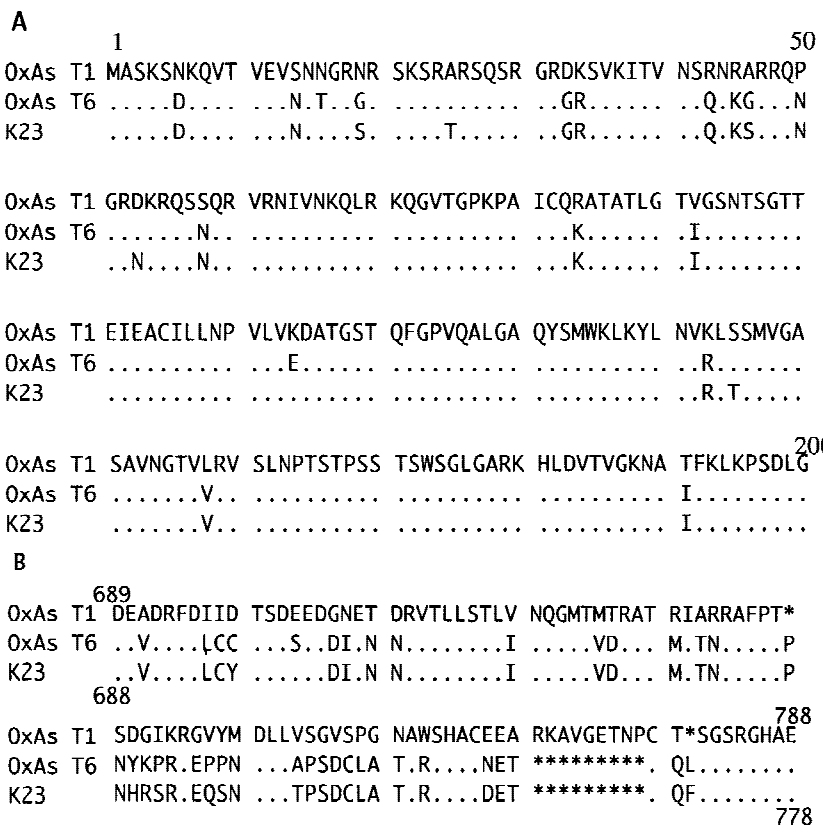

Fig. 1. Comparison of the predicted amino acid sequences of the ORF2 region of OxAs T6 and K23 with that of OxAs T1. **A:** N-terminal region; **B:** C-terminal region. Amino acids identical to OxAs T1 are indicated by a dot. Variable amino acids are shown by the appropriate single-letter amino acid code. Asterisks denote a deletion. Amino acid numbers from the ORF2 N-terminal of OxAs T1 are given at the top, and those of T6 are at the bottom.

acids) deletion from its nucleotide sequence (Fig. 1). The deletion was attributed to the position of the 3′ end of ORF2 (Fig. 1B). The identity of the 130 bp at the 3′ end of ORF1b connected to ORF2 between K23, K24, OxAs T1, and T2 was found to be very high (>92%). These results indicate that the ORF2 part of HAstVs has both a conservative and a variable region, on which we designed primers as well as developed an RT-PCR procedure to detect specifically all serotypes of HAstV.

Reverse Transcription–Polymerase Chain Reaction to Detect All Serotypes of HAstV

Extraction of viral RNA using ISOGEN-LS took only about 1 hr, because no complicated pretreatment steps were necessary. The AC4/AC6 primer set worked well in amplifying HAstV, with the exception of T4 (Fig. 2A). The products of OxAs T6 and K23 were about 20 bp smaller than those of the others (Figs. 1 and 2A). Our newly designed AC230/AC1′ primers amplified all serotypes (Fig. 2B), and the product could be confirmed by Southern hybridization with ACom-probe (Fig. 2C). Using AC230/AC1′ primers on the fecal specimen, with final concentrations of 0.5 mmol/L and 1 mmol/L of $MgCl_2$, a single, clear 230-bp band was seen; at higher concentrations of $MgCl_2$, the PCR products showed extra and/or smeared bands. The other new isolated strains from sporadic cases of diarrhea also could be detected using this primer set, and the products were confirmed by hybridization (Fig. 3). None of the other three control tested RNA viruses produced any products (Fig. 3, lanes 10–13), that is, the AC230/AC1′ primer set amplified only HAstVs. The detection of HAstV RNA by RT-PCR proceeded quickly, in about 5–6 hr from extraction to observation.

Multiplex Reverse Transcription–Polymerase Chain Reaction of HAstV and Norwalk-Like Virus

In Japan detection of NLV using RT-PCR is required when cases of food-borne gastroenteritis occur; at the same time, there has been almost no examination of HAstV, which is one of the causative agents of viral gastroenteritis [Oishi et al., 1994; Utagawa et al., 1994; Saito et al., 1995]. We decided to make good use of the NLV detection system to detect HAstV. We developed a one-tube RT-PCR to detect both viruses at the same time by multiplex RT-PCR. In experiments, the optimal concentration of AC230/AC1′ primers was found to be 0.2 μmol/L. Various combinations were tested: the PCR mixture containing both primer sets and either RNA (Fig. 4, lanes 2 and 3), both RNA and either primer set (lanes 4 and 5), and both RNA and primer sets (lane 1) amplified each product independently and specifically. Both HAstV and NLV were amplified using the respective suitable primers in a single PCR test tube, and each PCR product was obtained as a clear band at 230 bp (HAstV) and 330 bp (NLV), as shown in Fig. 4.

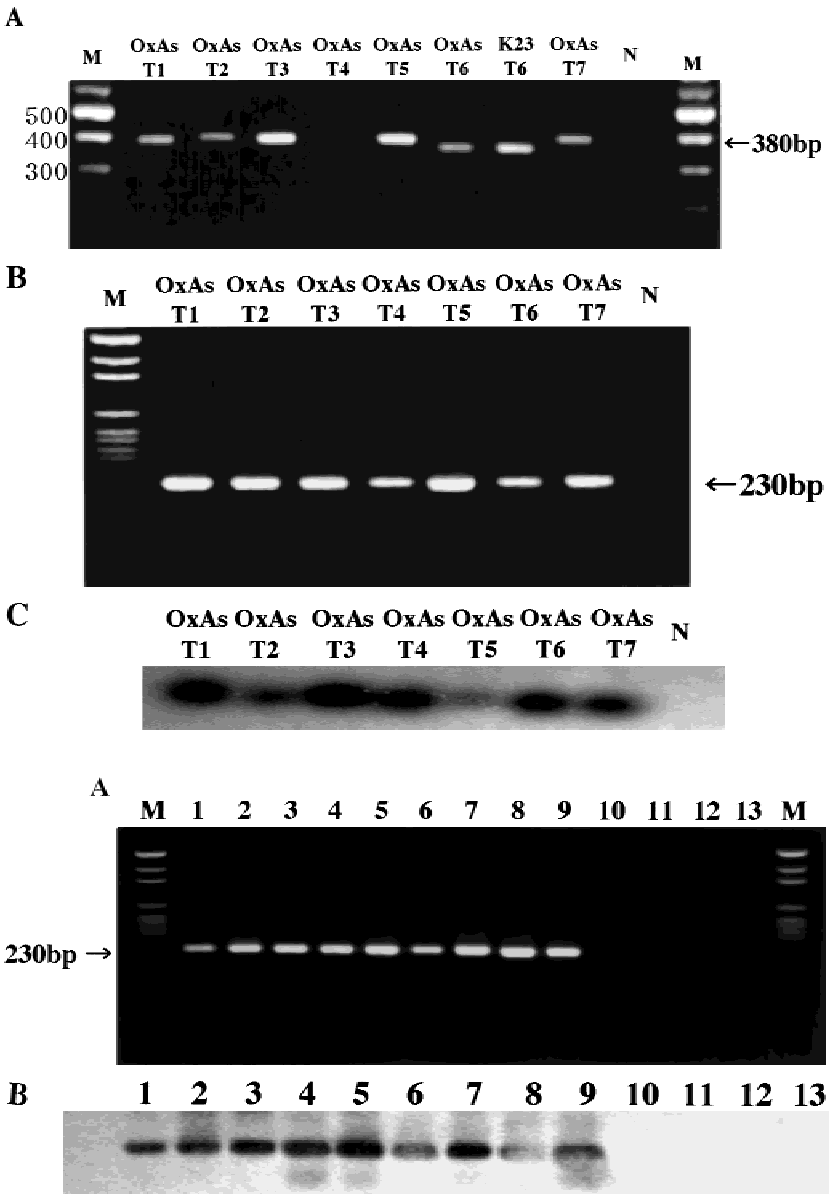

Fig. 2. Reverse transcription–polymerase chain reaction products from reference and K23 strains using two primer sets. Agarose gel electrophoresis of the PCR products, which are indicated by a header over the top of each lane. **A:** Using AC4/6 primer set. M, 100-bp DNA ladder molecular marker (MW: 300–600 bp). **B:** Using the AC230/AC1′ primer set. M, pGEM DNA molecular marker (MW: 222–2,645 bp). **C:** Southern hybridization with ACom-probe. N, negative control with water. The arrow indicates the 380 bp (A) and 230 bp (B) of PCR product.

Fig. 3. Application of field cases of diarrhea associated with HAstV in Japan and other viruses by RT-PCR with the AC230/AC1′ primer set. **A: Lane 1:** K23; **lane 2:** cultured K23 with CaCo-2 cell line; **lane 3:** K24; **lanes 4–9:** isolated strains from sporadic cases, serotypes 4, 5, 4, 4, 4, and 6; **lane 10:** rotavirus (SA11); **lane 11:** Norwalk-like virus (KY89/89/J); **lane 12:** hepatitis A virus (KRM238); **lane 13:** negative control with water; lane M: pGEM DNA molecular marker. The arrow indicates the 230 bp of PCR product. **B:** Southern hybridization with ACom-probe.

DISCUSSION

Katano virus HAstV T6 was identified as the causative agent of a large-scale outbreak of acute gastroenteritis that occurred in Katano City, Japan, in 1991 [Oishi et al., 1994]. To analyze the entire nucleotide sequence of the ORF2 HAstV T6, both K23 and K24 were selected as representative strains. When RT-PCR of Katano strains was carried out using the AC4/AC6 primer set, PCR products could be observed clearly, with sizes about 20 bp smaller than those of other serotypes (Fig. 2A). We were interested in the genome of Katano strains, especially the capsid-coding region ORF2. Nine PCR products making up the ORF2 nucleotide sequence were compared with other HAstVs. The sequence similarity between the OxAs T6 reference strain and the Katano strains indicated were well-conserved genomes, irrespective of time and place of virus isolation. The 99.8% nucleotide sequence identity between the Katano strains K23 and K24 provided conclusive evidence of the Katano virus outbreak from school lunches contaminated with this virus [Oishi et al., 1994]. After cell passages, however, the sequence changed, particularly in the 3′ end. The 3′ end of HAstV T6 ORF2 may be susceptible to mutation by cell passage. We are also interested in the alterations of virus genome and structure of HAstV T6 after cell passage, as previously described by Willcocks et al. [1990].

Genomic characterization of the NLV capsid protein showed that the most conserved region was found between amino acid residues 30 and 250, while the C-terminal region exhibited significant variation. The NLV capsid protein has a modular structure consisting of three domains: P1, which appears bilobed; P2, the central stem domain; and a shell domain called S. It

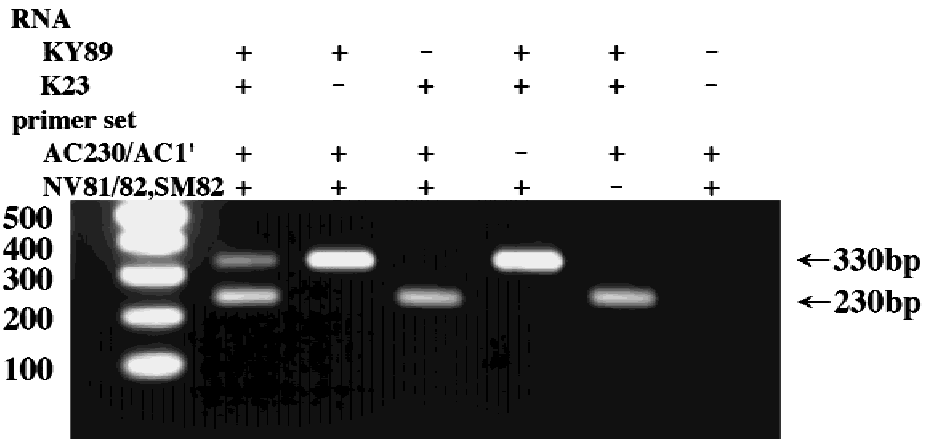

Fig. 4. Multiplex reverse transcription–polymerase chain reaction to detect HAstV and NLV. The RNA templates used were K23 and NLV (KY89/89/J), and the primer sets used were AC230/AC1′ and NV81/82, SM82. (+) and (−) indicate the presence and absence of the RNA template or the primer set, respectively. 100-bp DNA ladder molecular marker (fragments including 100–500 bp). The arrow indicates the 330 bp of the products of NLV and 230 bp of the products of HAstV.

was speculated that the S domain may constitute the conserved region, while the C-terminal variable region formed the P1 and P2 domains [Prasad et al., 1996]. The ORF2 region of HAstVs, including the Katano strains, showed a genomic characterization similar to that of NLV, with two different regions having unique sequence characteristics. The 5′ end between amino acid residues 1 and 385 was found to be very conserved in seven of the eight serotypes (except T7), as shown in Fig. 1A. The results of Willcocks et al. [1995] and Moceyron et al. [1997], as well as our own (shown in Fig. 1B), indicate that the region beyond 386 amino acid residues, up to the 3′ end, is highly variable. It is possible that the S domain of HAstV was formed by the N-terminal conserved region and the construct of the P domain was a C-terminal variable region.

Many PCR detection primers for HAstVs designed for the variable region at the 3′ end have been published [Jonassen et al., 1995; Noel et al., 1995; Saito et al., 1995], as well as our AC4/AC6 primer set. It is believed that PCR products of the 3′ end contain much information about sero- and genotyping, but it was not easy to design a primer set to detect all serotypes. To detect all serotypes of HAstV with RT-PCR, it seemed more practical to design detection primers for the conservative region. Based on this idea, we used the new AC230/AC1′ primer set designed from the conserved region.

This primer set could detect all HAstV serotypes, specifically amplifying a 0.5-μL RNA suspension. Preliminary tests resulted in a sensitivity of HAstV RNA detection using the AC4/AC6 primer set of 0.8 pg/μL, while AC230/AC1′ showed almost the same sensitivity. We used ISOGEN-LS to extract viral RNAs, since it was found to be easier and more rapid than the cetyltrimethylammonium bromide (CTAB) method. Comparing the sensitivity to detect NLVs using RT-PCR with these two different extraction methods, we noted no differences. Furthermore, multiplex RT-PCR to detect HAstV and NLV, which are important causal agents of acute gastroenteritis, is appropriate for rapid viral diagnosis of food-borne gastroenteritis. With this system, diagnosis can be carried out rapidly, in about 5–6 hr from RNA extraction to electrophoresis of PCR products, detecting both HAstV and NLV simultaneously in one test from fecal specimens of patients with gastroenteritis. Consequently, our method is more useful and rapid for the diagnosis than other methods [Jonassen et al., 1995; Noel et al., 1995; Mustafa et al., 1998].

Our study uncovered important information about the genetic characterization of human astroviruses and their epidemiologic significance. We have established a multiplex PCR for HAstV and NLV that can detect rapidly and specifically. A faster diagnostic system has been developed for the detection of HAstV T1 through T7 and NLV and should contribute to clarifying their epidemiologic features.

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